RT-PCR Detection of Tyrosinase, gp100, MART1/Melan-A, and TRP-2 Gene Transcripts in Peripheral Blood of Melanoma Patients

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Aim. To detect the expression of genes encoding tyrosinase, gp100, MART-1/Melan A, and tyrosinase-related protein-2 (TRP-2) in peripheral blood of melanoma patients by reverse transcription-polymerase chain reaction (RT-PCR).

Methods. Nineteen peripheral blood samples were obtained from 17 melanoma patients. When tested, 15 of them presented with clinically detectable metastatic disease. Samples of peripheral blood (7 mL) were collected from each patient into vacutainer cell preparation tubes. Mononuclear cells were isolated, total cellular RNA extracted, and then used as a template for reverse transcription to complementary DNA (cDNA). The cDNA was thereafter assayed by PCR for the expression of melanocyte-associated transcripts of tyrosinase, gp100, MART1/Melan-A, and TRP-2 genes.

Results. Gp100 gene expression was detected in 13 out of 19 samples. In 4 of them, TRP-2 gene expression was also detectable. Expression of tyrosinase and Melan-A/MART-1 genes could not be observed. Interestingly, gp100 and TRP-2 gene transcripts were detected in patients having recurrent and/or metastatic disease at the time of testing.

Conclusion. The results we obtained support the use of RT-PCR assay for indirect detection of melanoma cells in peripheral blood of melanoma patients. As the transcripts for the tyrosinase gene and MART-1/Melan A gene were not detected, additional optimization experiments of RT-PCR assay are required.

Key words: gene expression; melanoma; neoplasm circulating cells; reverse transcriptase polymerase chain reaction

Early detection of circulating tumor cells in the peripheral blood might represent a powerful tool for the identification of melanoma patients with probable tumor progression and potentially poor clinical outcome (1-4). The development of polymerase chain reaction (PCR) assays allows the detection of gene markers present at low copy numbers with a sensitivity significantly higher than that achieved by the use of antibody-based techniques (5-10). In 1991, Smith et al (11) suggested that melanoma cells could be indirectly detected in the peripheral blood with coupled reverse-transcription (RT) and PCR (RT-PCR) to amplify tyrosinase messenger RNA (mRNA). Because blood cells do not express tyrosinase gene, and melanocytes and melanoma cells are not known to physiologically circulate, the detection of tyrosinase transcripts in the peripheral blood of patients with melanoma has been considered an indirect evidence of circulating melanoma cells. Since this original report (11), other groups have tested tyrosinase gene expression alone or in combination with other genes (12-24), whose expression is restricted to melanocytes and/or melanoma cells (25). The data reported, although disparate regarding the sensitivity and correlation with patients' stage of disease and prognosis, indeed suggest a potential applicability of RT-PCR technique in indirect detection of melanoma cells in the blood circulation (12-24,26,27). The use of single-gene marker assays may be unreliable due to heterogeneity and variation in the level of individual gene marker expression, particularly in advanced stages of tumor progression. Accordingly, by using more markers one can expect improved sensitivity (14,15,17,19,24,27).

We assayed peripheral blood of 17 melanoma patients for melanocyte-associated transcripts derived from tyrosinase, gp100, MART1/Melan-A, and tyrosinase-related protein-2 (TRP-2) genes.

Material and Methods

Patients' Data and Clinical Characteristics

Nineteen peripheral blood samples from 17 melanoma patients were evaluated (Table 1). All patients had pathologically verified malignant melanoma. Fifteen patients presenting with recurrent and/or metastatic disease were admitted to the University Hospital for Tumors for further diagnostic examination and surgical or radiotherapeutical treatment. Two patients with no evidence of locoregional lymph node spread of melanoma or distant metastases (patients No. 16 and 17) were admitted to the Hospital for primary surgical treatment. A patient with clinically progressive disease and visceral metastases was tested three times...
within a period of several months, i.e., during each hospital admission for palliative radiation therapy treatment (patient No. 15).

All patients were informed about the purpose of the study and gave their consent. The study protocol was approved by the Ethics Committee of the Hospital.

Blood Collection

During routine collection of peripheral blood for standard diagnostic hematological and biochemical analyses, 7 mL of peripheral blood from each patient were collected upon hospital admission into vacutainer cell preparation tubes containing sodium citrate, Ficoll-Hypaque, and polyether gel (CPT vacutainer tubes, Becton Dickinson, Oxford, UK) allowing one-step isolation of lymphocytes, monocytes, and plasma (16,28). As controls, peripheral blood samples from two healthy donors and from two patients with myeloproliferative diseases (29,30) were also included in the analysis.

Blood samples were processed within 6 h after collection. Tubes were centrifuged for 20 min at 3,000 r.p.m. at room temperature. Buffy coats above the polyether gel were removed and washed with 15 mL phosphate-buffered saline (PBS); pellets were then resuspended in 1 mL PBS and transferred to microfuge tubes, spun, and lysed with the same volume of RNA extraction buffer containing 4 mol/L guanidium thiocyanate (28,31-33). Samples were then stored at -20°C.

Spiking Experiments of HBL Melanoma Cell Line

Established HBL melanoma cell line was kindly provided by Dr. C. C. Spagnoli, Department of Surgery, Division of Research, University Hospital Basel, Switzerland. Originally, this cell line was obtained from Dr. G. Ghanem, Free University of Brussels, Belgium. This cell line was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics. Tumor cells were maintained in a monolayer and passaged upon trypsinization. For spiking experiments cells were collected, washed with PBS, counted, and added in appropriate cell numbers to peripheral blood samples from healthy donors collected into CPT tubes. These spiked blood samples were then processed as described above.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from isolated peripheral blood mononuclear cells (PBMC) as described (28,31,32), by the guanidinium-isothiocyanate-phenol-chloroform method (33). Reverse transcription was carried out using Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (GeneAmp RNA PCR kit, Applied Biosystems, NJ, USA) (28,31-33).

The cDNA obtained was tested for the presence of defined gene sequences in PCR (GeneAmp kit; Perkin-Elmer-Cetus, Norwalk, CT, USA) performed in 50-μL volumes, using specific primer pairs. The following primers were used for PCR-reaction: β-actin sense, 5’-GAA ACT ACC TTC AAC TCC ATC-3’; β-actin anti-sense, 5’-TAG CAG GTG GCG TTT AGG AAC ATC-3’; yielding an amplification product of 317 base pairs; gp100 sense, 5’-GAC TCT GAT TAG TCG GAA ACT-3’; gp100 anti-sense, 5’-CAC CAA GTC GAC AAG AGC AG-3’, amplifying a 344-bp fragment; tyrosinase outer anti-sense, 5’-GCT ATC CCA GTA AGT GGA CT-3’; tyrosinase outer sense, 5’-GTC TTT ATG CAA TGG AAC GC-3’, amplifying a 364-bp fragment; MART-1/Melan-A anti-sense, 5’-AGA TGC CAA GAG AAG ATC-3’; MART-1/Melan-A sense, 5’-GCT CTT AAG GTG AAT GCC TAC CAG CCT GTC TAC-3’; amplifying a 334-bp fragment; 5’-TTG GCA GAT TGT CTC TAG GC-3’, amplifying a 345-bp fragment; tyrosinase outer sense, 5’-GCT CTT AAG GTG AAT GCC TAC CAG CCT GTC TAC-3’; tyrosinase outer anti-sense, 5’-AGG CAT TGT GCA TGC TGC TTC-3’, amplifying a 284-bp fragment; tyrosinase nested sense, 5’-GTC TTT ATG CAA GTC GAC GC-3’; tyrosinase nested anti-sense, 5’-GCT ATC CCA GTA AGT GGA CT-3’, amplifying a 207-bp fragment (11,28,34-36).

Amplication of tyrosinase, gp100, MART-1/Melan-A and TRP-2 gene transcripts required 35 cycles including a 45 s annealing step at 55°C and a 45 s extension at 72°C. Final extension at 72°C was prolonged for 7 min. Amplification of β-actin positive control transcripts required 30 similar PCR cycles.

RT-PCR products were run on 1.5% agarose gels in the presence of ethidium bromide. Gels were then photographed under ultraviolet transillumination. Molecular weight markers providing bands at 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118, and 72 base pairs (Gibco BRL Life Technologies Inc., USA) were included in all gels.

Results

Sensitivity of the RT-PCR Assay

To determine the sensitivity of the assay, we added serially diluted cells from the established melanoma cell line HBL into 7 mL samples of peripheral blood from a normal donor. Blood samples were spiked with 1.8x10^6, 1x10^5, 1x10^4, 1x10^3, 1x10^2, 1x10^1, or 1 HBL melanoma cell per sample (Fig. 1). Previous expression studies had revealed a relatively high expression of MART-1/Melan-A, gp100, and tyrosinase genes in this cell line (28). MART-1/Melan-A gene expression was studied in detail upon 45 cycles PCR. As shown in Figure 1, the expression of MART-1/Melan-A gene in samples containing as little as 1 to 10 HBL cells per blood sample could be detected (lanes 1 to 7). Complementary DNA from a nonspiked blood sample was used as a negative control. This sample was negative for MART-1/Melan-A gene expression (lane 8). In all samples, β-actin gene expression was highly evident (data not shown). Thus, these results indicate that the RT-PCR technique applied to normal donor blood samples spiked with melanoma line cells allows detection of melanoma cells.

Detection of Tyrosinase, gp100, MART-1/Melan-A and TRP-2 Gene Transcripts in Peripheral Blood

Results from RT-PCR testing of 19 blood samples from 17 melanoma patients, two healthy donors and two patients with myeloproliferative diseases are pre-
Table 1. Beta-actin, gp100, tyrosinase, MART-1/Melan-A, and TRP-2 gene expression in peripheral blood from patients with cutaneous malignant melanoma (n = 17) and controls (n = 4)

<table>
<thead>
<tr>
<th>Patient data</th>
<th>β-actin</th>
<th>tyrosinase</th>
<th>gp100</th>
<th>Melan-A/MART-1</th>
<th>TRP-2</th>
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<tbody>
<tr>
<td>Patients with recurrent and/or metastatic disease</td>
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<tr>
<td>1, SC, m, 45, pT1N0M0</td>
<td>+++</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2, MS, f, 38, TpTN1M0</td>
<td>+++</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3, KT, m, 65, pT3N0M0</td>
<td>+++</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4, SM, m, 68, pT1N1M0</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>5, BS, m, 54, TpTN0M0</td>
<td>++</td>
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<tr>
<td>6, JP, m, 58, TpTN0M0</td>
<td>++</td>
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<tr>
<td>7, JA, m, 20, TpN1pM0</td>
<td>++</td>
<td>–</td>
<td>+</td>
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<tr>
<td>8, MP-H, m, 50, pT1N0M0</td>
<td>+++</td>
<td>–</td>
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<tr>
<td>9, fC, m, 62, TpN0M0</td>
<td>+++</td>
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<tr>
<td>10, SM, m, 53, TpN0M0</td>
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<td>11, IP, m, 54, TpT1N0M0</td>
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<td>12, MD, f, 67, pT1N0M0</td>
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<tr>
<td>13, MP, f, 38, TpN1M0</td>
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<td>–</td>
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<td>14, IC, f, 57, TpN1M0</td>
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<td>–</td>
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<tr>
<td>15, JA, m, 20, TpN1pM0</td>
<td>+++</td>
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<tr>
<td>Patients surgically treated for localized melanoma</td>
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<tr>
<td>16, LJB, f, 32, pT1N0M0</td>
<td>+++</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>17, MV-M, f, 43, pT1N0M0</td>
<td>+++</td>
<td>–</td>
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<tr>
<td>Controls</td>
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<td>a, patient with myeloproliferative disease</td>
<td>+++</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>b, patient with myeloproliferative disease</td>
<td>+++</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
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<tr>
<td>c, healthy donor</td>
<td>+++</td>
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<tr>
<td>d, healthy donor</td>
<td>+++</td>
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</tr>
</tbody>
</table>

Patient data include initials, gender (male/female), age (years), and TNM status (American Joint Committee on Cancer; ref. 4). The symbol “++ “ indicates “very strong” or “strong” detection of specific gene transcripts on a 1.5 % agarose/ethidium bromide gel following 45 cycles of reverse transcription-polymerase chain reaction (RT-PCR) amplification (fat bands of β-actin transcripts). The symbol “+” indicates relatively weak but still visible amplification products. The symbol “+/–” indicates visible weak faint band. The symbol “–” indicates undetectable gene expression.

The two normal blood donors tested negative for tyrosinase, gp100, MART-1/Melan-A, and TRP-2 gene transcripts. For example, undetectable expression of gp100 gene is shown in Figure 2. Two patients with myeloproliferative disease tested weakly positive for gp100 gene expression (Fig. 3) but negative for tyrosinase, MART-1/Melan-A and TRP-2 gene transcripts (not shown).

Regarding melanoma patients, gp100 gene expression was detected in 13 out of 19 samples. TRP-2 gene expression was detected in 4 samples, which were also found to be gp100 positive (Table 1). Expression of tyrosinase and Melan-A/MART-1 genes could not be observed in any patient. Interestingly, expression of gp100 and of TRP-2 gene was detected in patients having recurrent and/or metastatic disease at the time of testing.

One patient (No. 15) with distant progressive visceral metastatic disease was tested on three different occasions within a period of several months. Expression of gp100 gene was reproducibly detected in all blood samples, whereas TRP-2 gene transcripts were detected twice.

Representative RT-PCR analysis of gene expression in the peripheral blood from seven melanoma patients is shown in Figure 4. Five of them tested positive for gp100 gene expression (patients No. 8, 9, 11, 13, and 14), whereas two out of seven tested negative (patients No. 12 and 17). A gp100-positive patient (No. 13) tested also positive for TRP-2 gene expression. All seven patients tested negative for tyrosinase and Melan-A/MART-1 gene expression. Further testing of tyrosinase gene expression in some patients with “nested” RT-PCR primers (11) was also negative (Fig. 5).

Figure 2. Undetectable expression of gp100 gene transcripts in peripheral blood from two normal donors. Lane 1 represents negative control, ie, reverse transcription-polymerase chain reaction (RT-PCR) amplification without the target cDNA molecules. Lane 2 and 3 represent peripheral blood samples from two normal donors. Lane 4 represent peripheral blood from the patient No. 15. Molecular weight markers are in lane “m” and provide 100 base pair ladder.
Discussion

Early detection of malignant disease is likely to ensure more effective tumor control and, consequently, better prognosis for these patients. RT-PCR techniques potentially represent highly sensitive methods for the detection of limited numbers of cancer cells. The clinical relevance of these assays as tools for the detection of malignant cells is currently actively investigated (6-10).

Tyrosinase, an enzyme responsible for the first two steps of melanin biosynthesis, is one of the most specific markers in melanocytic differentiation, expressed only by melanocytes, melanoma cells, and Schwann cells (25). Although the detection of tyrosinase gene transcripts in peripheral blood has been suggested to indicate the presence of circulating melanoma cells (11), its clinical relevance is still unclear (8,12,14,21,26,27). High degree of result variability has been demonstrated by several authors, probably due to differences between various RT-PCR protocols (26,27). The most striking discrepancies were seen in patients with stage IV disease, where sensitivity rates ranged from 0% (17) to 100% (13). False positive results have been imputed to contamination with normal melanocytes during blood collection, whereas tyrosinase mRNA degradation might lead to false negative results (16,18,27). In addition, variability in tyrosinase expression in melanoma patients has been explained by several authors as due to intermittent presence of tumor cells in the peripheral circulation, heterogeneity of tumor cells with variation in the level of gene marker expression, and/or presence of tumor clones not expressing tyrosinase gene (12,23,24). Considering this variability, indirect detection of melanoma cells by RT-PCR still appears to require technical improvements and standardization (12,15,16,27). Moreover, a multiple-marker RT-PCR assay seems to be more reliable and sensitive than a single-marker assay for the detection of melanoma cells in the blood (14,15,17,19,24,27).

We tested four different melanocytic marker genes: tyrosinase, gp100, MART1/Melan-A, and tyrosinase-related protein-2 (TRP-2). In addition, the expression of gp100 gene can also be found in tumor samples and cell lines of nonmelanocytic origin (24). All these genes specify also for melanoma-associated tumor antigens, which are commonly recognized by T lymphocytes. Therefore, the detection of tumor cells in the peripheral blood can also add to planning of antigen-specific immunotherapy in melanoma patients (3,4,19,28,32). Expression of β-actin gene was detected in all samples, thus testifying the integrity of cDNA preparations. Our results indicate that expression of melanocytic lineage genes is detectable by RT-PCR in blood samples spiked with melanoma cells from normal donor, used as positive controls. As expected, in two blood samples from normal blood donors the expression of these genes was undetectable. Weak expression of gp100 gene but not of tyrosinase, MART1/Melan-A, and TRP-2 gene, was detected in two samples from patients having myeloproliferative diseases, indicating thus "genomic instability" and/or illegitimate transcription in their blood cells (24).

Comparative analysis of gene expression in peripheral blood from our group of melanoma patients resulted in the amplification of gp100 and TRP-2 but
not of tyrosinase and of MART1/Melan-A gene transcripts. Expression of gp100 gene was detected in 11 out of 15 patients and in four of them expression of TRP-2 gene was also observed. These inconsistencies in gene expression could arise from low numbers of target molecules in the RNA samples and/or also be related to their poor quality, unsuitable for reliable detection of the tyrosinase and MART1/Melan-A genes. To exclude the possibility of false-positive results, due to possible gp100 gene expression in non-melanocytic cells (14,24), additional markers should be included into multimarker PCR studies aimed at increasing diagnostic and prognostic value of such tests in melanoma patients. Consistent detection of β-actin gene expression contradicts with the assumption of poor quality of RNA samples but it should be kept in mind that β-actin gene is so abundantly expressed that it can be detected even in poor quality mRNAs and cDNAs (27). Remarkably, in patient No. 15, who was tested at three different time points, we could detect gp100 gene expression three times and TRP-2 gene expression twice. In two blood samples from patients with clinically non metastatic disease no specific transcripts could be detected.

Results from our pilot study support the use of RT-PCR assay in the indirect detection of melanoma cells in peripheral blood of melanoma patients. Evaluation of the clinical relevance of RT-PCR detection of circulating melanoma cells in patients still requires further studies. It is possible that most circulating melanoma cells do not have full metastatic potential, ie, only a few cells will complete the multistep process of selection leading to metastasis (12). Moreover, the lack of tyrosinase mRNA detection in the blood does not necessarily exclude metastatic progression, since circulating and/or metastatic melanoma cells could be intermittently present in the peripheral circulation (12,23,26). Also, circulating melanoma cells might be heterogeneous in the expression of molecular markers, giving false negative results (12,23). On the other hand, in patients with progressive metastatic disease, survival was reduced in RT-PCR tyrosinase positive as compared with RT-PCR tyrosinase negative patients (21,23).

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References


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